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and Breast Cancer Risk in African-American Women

PRINCIPAL INVESTIGATOR: Christine Ambrosone, Ph.D.

CONTRACTING ORGANIZATION: National Center for Toxicological
Research
Jefferson, Arkansas 72079-9502

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FOREWORD

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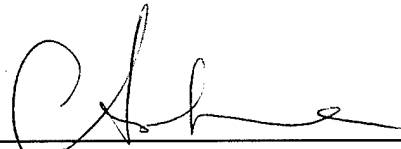
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Introduction

Among African-American women younger than age 50, breast cancer incidence is almost twice that of Caucasian women. African-American women are more often diagnosed with aggressive tumors and have higher mortality rates than Caucasians. Differences in tumor biology and mortality do not appear to be due to factors related to socioeconomic status. Little is known regarding explanations for these racial disparities, perhaps because of the difficulty in enrolling African-Americans into research studies. The purpose of this pilot study was to develop a novel method of recruitment, focused primarily on minority women, and investigate previously unexplored risk factors in breast cancer epidemiology. Through rapid case ascertainment by tumor registries in Arkansas, we intend to enroll approximately 260 cases over two years, frequency-matched to controls randomly selected from Health Care Finance Administration (HCFA) and Arkansas Driver Services (ADS) lists. Cases and controls are matched to racially similar breast cancer survivor-recruiters. Potential participants are sent introductory postcards with the recruiters' photographs on them. Several days later, the recruiters call the potential participants to describe the study and seek their participation. Culturally appropriate interviewers administer questionnaires, draw blood and collect urine specimens from the participants. Once processed, data from these sources will be used to explore study hypotheses related to gene/environment interactions. We intend to evaluate the role that diet particular to African-Americans in the rural South may play in breast cancer etiology, and to assess the possible modification of risk by genetic differences in steroid hormone and carcinogen metabolism. A specimen bank was established to enable exploration of future hypotheses.

Body

The proposed work was a pilot case-control study of breast cancer in African-American women. We realized, however, that without a comparable Caucasian group from the same locales as the African-American women, interpretation of the data would be difficult. It would be impossible to determine if specific risk factors are more prevalent in African-American women and are, thus, related to the increased early age at onset and more aggressive disease, or if they are merely regional habits that are shared by women of both groups. Therefore, additional funding was sought from the Public Health Service Office of Women's Health (DHHS PHS OWH) to support an identical study in Caucasian women, so that results could be compared.

Research accomplishments associated with each Task outlined in the Statement of Work will be addressed within the context of each of the accomplishments.

Technical Objective 1 Develop and pilot a novel approach for enrolling minority women into research studies.

Task 1: Months 1-2: Organizational start up tasks--finalize questionnaire, continue training sessions and role-playing with Witness ProjectTM recruiters and interviewers.

These tasks were accomplished in year 1. The questionnaire was finalized, interviewers are well-trained and experienced, and the recruiters are highly successful. Meetings are held regularly with recruiters to maintain enthusiasm and commitment, and to troubleshoot areas of difficulty. A paper detailing the recruitment strategies, 'Challenges, Limitations and Strategies for Increasing Participation in Epidemiologic Studies', was rewritten in response to reviewers from *Cancer Epidemiology, Biomarkers and Prevention*, and has been resubmitted. In the spring of 2000, a presentations were made regarding recruitment methodology in our study at a Keystone Conference in Taos entitled "*Molecular Epidemiology: A New Tool in Cancer Prevention*". It was also presented in poster format at the Annual Meeting of the American Association for Cancer Research. Within the last year, one of our most dedicated recruiters, Ms. Linda Creggett, lost her fight with metastatic breast cancer. She is sorely missed. Two more recruiters were trained and are now successfully working in the field in the last year.

Task 2: Months 3-24. Identify incident breast cancer cases by rapid ascertainment; Identify controls from Department of Motor Vehicles and State Identity lists; recruitment of 230 cases and 230 controls by staff from Witness ProjectTM. Periodically assess effectiveness of individual recruiters by evaluation of response rates among women contacted by each individual.

Recruitment of both cases and controls is ongoing. The study has not moved as quickly as anticipated, primarily because of the inability to identify sufficient numbers of African-American women with breast cancer who are eligible for the study. Seventy-five percent of African-American Arkansan women who are diagnosed with breast cancer live in either Pulaski or Jefferson County. We currently identify cases through the Arkansas Cancer Research Center, the major cancer treatment facility in Pulaski County. A large

number of African-American women with breast cancer who reside in Southeastern Arkansas receive treatment at Jefferson Regional Medical Center (JRMC). While JRMC physicians agreed to allow us to contact their patients in December, 1996, access to tumor registry information was not forthcoming. Problems were first encountered with the JRMC Tumor Registrar, who was reluctant to release patient names. This situation was complicated by the fact that JRMC has no OPRR-approved Institutional Review Board. Thus, months were spent finalizing a Single Project Assurance with the OPRR and wrangling with legal concerns regarding access to patient names. All of these problems have been successfully overcome, however, and we are actively recruiting from JRMC. This still did not provide an adequate pool of eligible African-American women with breast cancer.

Two other approaches were taken to identify additional African-American women with breast cancer. It appears that a significant number of women who live in Northeastern Arkansas in areas bordering the Mississippi River (the state boundary) seek treatment in Memphis, Tennessee, rather than travelling to central Arkansas. We are collaborating with physicians at the Methodist Healthcare Cancer Center (MHCC) in Memphis, Tennessee. They have agreed to allow us to contact women from Arkansas who seek breast cancer treatment at their Memphis facilities. Methodist Healthcare owns and operates 16 hospitals in West Tennessee and Central Mississippi and is the largest private, not-for-profit hospital in the country. It is also ranked number one in market share (38.3%) in Memphis according to the 1996 Joint Annual Reports of Hospitals. With hospitals in all four corners of Memphis, approximately 90 Arkansan women with breast cancer seek treatment at MHCC each year. We are also working with physicians in the Arkansas Department of Health Breast and Cervical Cancer Screening Program. The ADH Medical Director, Dr. David Bourne, refers women who are diagnosed with breast cancer through their screening program to our study.

These sources did not yield the anticipated number of African-American women. Since the inception of the study, we have been knocking at closed doors of two other institutions, major hospitals in Little Rock that share the majority of breast cancer cases in Pulaski County. After refusal of their IRBs to give us access to patient names, we began contacting breast cancer surgeons in Little Rock. Relationships have been formed with 3 primary surgeons, one of whom led another, this time, successful, application to the IRB of Baptist Memorial Medical Centers in Little Rock and North Little Rock. Medical records from the 3 participating surgeons have yielded 226 eligible patients (diagnosed within the last six months, no prior cancers). Approximately 15% of those women are African-American. We are in the process of accessing records from the Baptist Registries, and anticipate that our numbers will greatly increase.

Due to unexpected programming difficulties with ADS and request processing difficulties with HCFA, control identification was been delayed. Over the last year, however, we have focused on control ascertainment and recruitment, while making diligent efforts to contact African-American women with breast cancer.

Table 1 shows numbers of women enrolled into the study to date, and response rates for both African-American and Caucasian women. To date, interviews have been completed for 385 women, aged 29-75, 248 with breast cancer and 137 community controls. The participation rate (the proportion of women who complete the study) for cases is 76% for Caucasian women, and 61% for African-American women. These rates are much improved over those using the standard methodology employed in an earlier study in this difficult-to-reach community, in which, for Caucasians and African-Americans (men and women) combined, participation rates were 37% and 30% for cases and controls, respectively.

Table 1. Participation in Case-Control Study

African-American Women			
	Total contacted	Number Enrolled	Participation Rate
Cases	79	50	63%
Controls	34	19	56%
Caucasian Women			
	Total contacted	Number Enrolled	Participation Rate
Cases	265	198	75%
Controls	155	118	76%

Task 3: Months 24-30

Technical Objective 2 With a Food Frequency Questionnaire (FFQ) supplemented with foods commonly eaten by African-American women in the rural south, investigate the role of dietary sources of fat and heterocyclic amines in BC risk.

Task 1: Months 1-3 Adapt FFQ to include foods found to be commonly eaten by African-American women in Eastern Arkansas previously surveyed.

As reported in the Annual Report for 1999, to determine if additional foods should be added to the Gladys Block Health Habits and History Questionnaire (HHHQ) to improve its suitability for African-American women in the lower Mississippi Delta, we conducted a survey of foods and cooking methods that may be particular to these residents. In collaboration with the Department of Dietetics and Nutrition at UAMS, a list of 60 foods commonly eaten by this population, such as wild game, parts of animals not traditionally eaten, and foods cooked with fat, was compiled through in-depth interviews and focus groups. We developed a Food Frequency Questionnaire with those foods elicited and then surveyed approximately 400 African-American women, aged 40 to 70, who live in eastern Arkansas. The survey indicated that few of the foods queried were eaten frequently by a large proportion of the population, but that several food items not on the Block questionnaire were eaten 1 to 4 times or more per month by > 50% of women surveyed. These foods included okra, southern peas (crowder, purple hull, split), butter and northern beans. Furthermore, more than 50% of women added fat when they cooked beans or greens, such as collards, mustard greens or kale. These additional items were added to the questionnaire already validated in Atlanta for a southern African-American population.

Task 2: Months 3-26 Interviews with cases and controls; ongoing monitoring of interviewers.

As stated above, interviewing of cases and controls is ongoing. Completed questionnaire booklets are reviewed weekly by the project director, for accuracy and coherence. Interviewer performance is thus evaluated continually. One phenomenon that we have observed using this methodology is the case in which a potential participant will agree when speaking with the recruiter, but then refuse when contacted by the interviewer. Late refusals may occur at the time the interview is scheduled, or after the interview is scheduled, often after several requests from the participant to reschedule. Interestingly, the pattern of late refusals varies by interviewer, and steps are being taken to train all interviewers in how to approach the potential participants who have already agreed to participate. We have also taken steps to cut down on the amount of time between the recruiter contact and the interview scheduling. Late refusal rates have dropped 20% since these changes were implemented.

Task 3: Months 24-30 Double data entry, with ongoing quality control.

Although this task was not targeted for until the beginning of year 3, we have developed a data base for entry of the questionnaire data. All data collected to date, have been double-entered and we are currently working on data checks and data cleaning for data that has been collected and entered. Interview data will be entered on an ongoing basis.

Technical Objective 3 Evaluate genetic variability in metabolism of HAs by examining phenotypic variability in CYP1A2 and sulfotransferase activity, as well as genetic polymorphisms in *NAT1* *NAT2*, *ST1A3* and *CYP1A2*.

Task 1: Months 3-26 Perform phenotyping assays for CYP1A2, NAT2, and phenol sulfotransferase.

These assays are ongoing, although analysis will not be evaluated by cases and controls until all data are collected. We did, however, use data for sulfotransferase, along with that from participants in a study of colorectal cancer, to evaluate correlations between data from the phenotyping and genotyping. The manuscript based on that data is in press in Pharmacogenetics (see Appendix).

Task 2: Months 26-30 Perform DNA analysis for genetic polymorphisms in *CYP1A2*, *NAT1*, *NAT2*, *ST1A3*

We established a biologic specimen bank in the context of this study, and protocols for processing and storage of blood were developed. Blood samples are processed so that there are aliquots of serum, plasma, platelets, red blood cells, and buffy coat. Using a processing system currently used in the 350,000-person EPIC study in Europe, each blood component is mechanically aliquotted into several 0.5ml straws that are prestamped with an ID number and barcode. Straws are heat-sealed and stored in canisters in liquid nitrogen tanks, with a detailed computerized mapping scheme in place. Our laboratory routinely performs high throughput genotyping and has extensive experience in assaying all of the genes proposed for study.

Task 3: Months 31-36

Key Research Accomplishments

- Establishment of infrastructure for molecular epidemiologic study (questionnaire development, protocols and equipment for blood processing and specimen banking, recruiter and interviewer hiring and training, development of data bases for participant tracking and questionnaire data, etc.), data entry.
- Enrollment of cases and controls into study – response rates far superior to those in earlier case-control study in the same locales.
- Adaptation of FFQ to the African-American population in Arkansas.

Reportable Outcomes

Presentations at Keystone Symposium “*Molecular Epidemiology: A New Tool in Cancer Prevention*” February 2000, Taos, NM and at the Annual Meeting of the American Association for Cancer Research - **Ambrosone CB**, Erwin DO, Morris-Chatta R, Long S. Patient advocate/survivors as recruiters to epidemiologic studies in a medically-underserved community. *Proc Amer Assoc Cancer Res* 2000; 41.

Manuscript in press:

Nowell S, Ambrosone CB, MacLeod SL, Mrackova G, Williams S, Plaxco J, Ozawa S, Kadlubar FF, Lang NP. Relationship of phenol sulfotransferase (SULT1A1) genotype to sulfotransferase activity in platelet cytosol. *Pharmacogenetics*, (in press).

Biologic specimen bank established with DNA, serum, plasma and red blood cells from cases and controls.

Grant submitted to NCI 6/2000, ‘Racial Patterns in Breast Cancer Etiology and Pathology’ Investigators: Ambrosone, Fontham, Erwin, et al.

Conclusions

In this pilot study, case ascertainment has been accomplished through collaborations with physicians at the Arkansas Cancer Research Center (ACRC) in Little Rock; Jefferson Regional Medical Center (JRMC) in Pine Bluff, Arkansas; Methodist Healthcare Cancer Center (MHCC) in Memphis, Tennessee and through the Arkansas State Department of Health. Most recently, we have received the cooperation of three additional breast surgeons, practicing at 3 different large hospitals in Little Rock and access to Tumor Registries of two of those hospitals. Controls have been identified through the Health Care Finance Administration and Arkansas Driver Services enrollment files. Interviews have been conducted using a questionnaire adapted from one developed by John Potter and Kristen Anderson at University of Minnesota, and used in a study of colorectal cancer in Arkansas (Lang, PI). This instrument was modified to collect data on breast cancer risk factors using the validated questionnaires from the Western New York Diet Study and the Women's Health Initiative. Blood and urine samples for genotyping and phenotyping have also been obtained. We have established a biologic specimen bank, with a detailed protocol for blood processing and storage. All of the assays to be performed have been refined in our laboratories at the ACRC and National Center for Toxicological Research (NCTR). Recruitment is well underway and the methodology appears to be a vast improvement over previous work in this area.

Full-scale epidemiologic studies require large budgets, which include personnel, supplies, equipment, etc. Furthermore, building of an infrastructure is essential, yet laborious and time-intensive. There was little to no organized epidemiologic studies being conducted in Arkansas when this study was initiated, and funding received from the Department of Defense and the OWH has been used to develop the infrastructure and get it the study into the field. We now have cooperation from several major and physicians and expect to more quickly ascertain African-American women diagnosed with breast cancer in the coming year. Our methodology has been established and tested, staff training manuals have been developed and successfully piloted, and a specimen bank has been established and is in use.

Appendix:
Manuscript in press

Relationship of Phenol Sulfotransferase (*SULT1A1*) Genotype to Sulfotransferase Activity Phenotype in Platelet Cytosol

Susan Nowell^{1,5}, Christine B. Ambrosone^{1,2}, Shogo Ozawa⁴, Stewart L. MacLeod¹, Gabriella Mrackova², Suzanne Williams¹, Jason Plaxco¹, Fred F. Kadlubar² and Nicholas P. Lang^{1,3}

¹University of Arkansas for Medical Sciences, Surgical Oncology Department, 4301 W. Markham St., Slot 725, Little Rock, AR 72205; ²Division of Molecular Epidemiology, National Center for Toxicological Research, Jefferson, AR; ³Central Arkansas Veteran's Health Care System, Little Rock, AR 72205 and ⁴Division of Pharmacology, National Institute of Health Sciences, Tokyo 158, Japan

⁵To whom correspondence should be addressed:

Susan Nowell
University of Arkansas for Medical Sciences
VA Research Slot 151
4300 W. 7th Street
Little Rock, AR 72205

Phone: (501)257-4808

Fax: (501)257-4822

e-mail: nowellsusana@exchange.uams.edu

Running title: SULT1A1 genotype-phenotype correlation

ABSTRACT

Sulfation catalyzed by human cytosolic sulfotransferases is generally considered to be a detoxification mechanism. Recently, it has been demonstrated that sulfation of heterocyclic aromatic amines by human phenol sulfotransferase (SULT1A1) can result in a DNA binding species. Therefore, sulfation capacity has the potential to influence chemical carcinogenesis in humans. Thus far, one genetic polymorphism (Arg²¹³His) has been identified that is associated with reduced platelet sulfotransferase activity. In this study, data on age, race, gender, SULT1A1 genotype and platelet SULT1A1 activity were available 279 individuals. A simple colorimetric phenotyping assay, in conjunction with genotyping, was employed to demonstrate a significant correlation ($r=.23$, $p<0.01$) of .between *SULT1A1* genotype and platelet sulfotransferase activity toward 2-naphthol, a marker substrate for this enzyme. There was also a difference in mean sulfotransferase activity based on gender, (1.28 nmol/min/mg, females; 0.94 nmol/min/mg, males, $p=0.001$). DNA binding studies using recombinant SULT1A1*1 and SULT1A1*2 revealed that SULT1A1*1 catalyzed *N*-OH-ABP DNA adduct formation with substantially greater efficiency (5.4 vs. 0.4 pmol bound/mg DNA/20 min) than the SULT1A1*2 variant. A similar pattern was observed with *N*-OH-PhIP (4.6 vs. 1.8 pmol bound/mg DNA/20 min).

INTRODUCTION

Human cytosolic sulfotransferases (SULTs) catalyze the transfer of the sulfonyl moiety from 3'-phosphoadenosine 5'-phosphosulfate (PAPS) to the hydroxyl, sulfhydryl, amino or *N*-oxide groups of a variety of endo- and xenobiotics (Jakoby and Ziegler, 1990). This reaction generally results in more polar metabolites, thus facilitating their elimination from the organism. At least eight SULT isoforms have been identified in humans (Her et al., 1995; Her et al., 1997; Her et al., 1998; Raftogianis et al., 1996; Sakakibara et al., 1998; Weinshilboum et al., 1997; Wood et al., 1994; Wood et al., 1996) and can be divided into two subfamilies, the phenol SULTs and the hydroxysteroid SULTs. SULT enzymes have a widespread tissue distribution and are expressed in the liver, lung, brain, skin, breast, kidney, gastrointestinal tissue, and, of significance to molecular epidemiologic studies, in blood platelets (Cappiello et al., 1990; Falany and Falany, 1996; Hume et al., 1996; Kudlacek et al., 1995; Zou et al., 1990). Platelets express SULT1A1 ("thermostable" sulfotransferase) and SULT1A3 ("thermolabile" sulfotransferase) (Anderson et al., 1998; Frame et al., 1999). In the case of SULT1A1, the activity found in platelets correlates to that found in other tissues (Coughtrie, 1996; Young et al., 1985) and is thought to be coordinately regulated. Platelet SULT activity has a strong genetic component, as evidenced by the substantial interindividual variability in the expression of this enzyme and its heritability, which has been estimated between 0.83 and 0.96 (Reveley et al., 1982).

Although traditionally considered Phase II detoxification enzymes, SULTs have also been implicated in the bioactivation of dietary and environmental procarcinogens (Glatt, 1997; Miller, 1994). Following *N*-oxidation by hepatic cytochromes P450,

sulfation of a variety of *N*-hydroxylated arylamines and arylamides produces reactive esterified metabolites (Abu-Zeid et al., 1992; Chou et al., 1995; Gilissen et al., 1994). We have shown that several *N*-hydroxy metabolites of arylamine and heterocyclic amine carcinogens, including *N*-OH-PhIP, can be activated to DNA binding species via sulfation by *SULT1A1* (Chou et al., 1995; Chou et al., 1995; Ozawa et al., 1994). Exposure to dietary and environmental procarcinogens has been strongly linked to the development of urinary bladder and gastrointestinal cancer (Anderson et al., 1997; Gonzalez, 1995; Massaad et al., 1992; Windmill et al., 1997). Therefore, genetic and phenotypic factors involved in interindividual variability of *SULT1A1* activity potentially plays a key role in individual susceptibility to disease.

Recently, genetic polymorphisms have been identified in *SULT1A1* (Ozawa et al., 1998; Raftogianis et al., 1997). In particular, one *SULT1A1* polymorphism results in an amino acid change (Arg to His, designated *SULT1A1**1 and *SULT1A1**2) at the conserved residue 213. In a predominantly Caucasian population, the frequency of the polymorphic allele was 0.674 and 0.313 for *SULT1A1**1 and *SULT1A1**2, respectively. The *SULT1A1**2 allele was associated with reduced sulfotransferase activity and thermostability in platelets, although the relationship to activity in human liver cytosol was not clear (Ozawa et al., 1998; Raftogianis et al., 1997). Coughtrie, *et al.*, (Coughtrie et al., 1999) investigated the frequency of this allele in both a Caucasian and Nigerian population and found no significant differences, in contrast to other drug metabolizing enzymes where there is considerable ethnic variation (Akullu et al., 1996; Kalow, 1991; Probst-Hensch et al., 1995; Stephens et al., 1994; Zhao et al., 1994). In this study, we employed genotyping along with a simple colorimetric activity assay to

evaluate the degree to which the *SULT1A1* genetic polymorphism correlates with platelet SULT activity toward 2-naphthol. Since the assay is designed to reflect the catalytic activity of only SULT1A1, these findings demonstrate the utility of genotype analysis as a contributor to SULT1A1 phenotype.

MATERIALS AND METHODS

Study participants

These data were derived from ongoing case-control studies designed to investigate the role of genotypic and phenotypic variability in modification of risk of colorectal and breast cancer associated with dietary heterocyclic amines and steroid hormones. Participants included those diagnosed with incident, primary, histologically confirmed cancer of the colon/rectum or breast, as well as community controls. Controls were frequency matched to cases on race, age and county of residence. Exclusion criteria for the case-control study included a history of cancer (other than non-melanoma skin cancer), uncontrolled cardiovascular disease, hepatic dysfunction as determined by bilirubin > 1.5 mg/dL, SGOT > 40 U/L, alkaline phosphatase > 140 U/L, and abnormal renal function as determined by BUN > 20 mg/dl and serum creatinine > 1.8 mg/dL. After obtaining informed consent from each participant according to an institutional review board approved consent protocol, a 24 ml blood sample was collected in four Vacutainer tubes (Becton Dickinson, Fisher Scientific, Houston, TX) containing ACD (ascorbate citrate dextrose) to prevent platelet aggregation. Blood samples were stored at room temperature until processed and platelets were isolated from other cell types within 24 hours of collection. Genotyping and phenotyping data were available for 279 participants.

Materials

Histopaque 1119 and 1077, 4-nitrophenyl sulfate (pNPS), 3'-phosphoadenosine 5'-phosphosulfate (PAPS), calf thymus DNA (Type I) and 2-naphthol were obtained from Sigma Chemical Company (St. Louis, MO). Other reagents used in the formulation of buffers were purchased from Sigma and were of the highest quality available. *Hae*II was purchased from New England Biolabs (Beverly MA). Taq DNA polymerase, along with other PCR reagents was purchased from Promega (Madison, WI). Metaphor agarose was obtained from FMC Bioproducts (Rockland, ME). DNA was extracted from lymphocytes using the Wizard genomic DNA isolation kit (Promega, Madison, WI). DNA was quantified using UV spectrophotometry, and its purity was determined by the ratio of its absorbance at 260 nm versus 280 nm. [2,2'-³H]N-OH-ABP (115 mCi/mmol) was prepared from [³H]4-nitrobiphenyl (ChemSyn Science Laboratories, Lenexa, KS) by reduction with ammonium polysulfide as described (Thissen et al., 1980). Radiolabeled [ring-³H]PhIP (88 mCi/mmol) was purchased from Chem-Syn Science Laboratories (Lenexa, KS). The nitro derivative of PhIP was synthesized according to the method of Grivas (Grivas, 1988) with the modifications made by Turesky, *et al.* (Turesky et al., 1991).

Platelet and lymphocyte separation

Individual blood cell components were isolated by centrifugation on discontinuous gradients of Histopaque-1077 and Histopaque 1119, using a modification of the manufacturer's protocol (Sigma Procedure No. 1119) as described by Frame, *et al.* (Frame et al., 1999). After separation, platelets were suspended in buffer (0.25 M sucrose, 10 mM triethanolamine, 5 mM 2-mercaptoethanol, pH 7.4) at a concentration

of 1×10^8 cells/ml. To ensure that the assays reflected activity in platelets and not contaminating cell types, an aliquot was analyzed for confirmation of platelet purity, using a Model STKS Coulter counter (Coulter Corp., Irving, TX). Isolated platelets routinely showed negligible contamination with other cell types (<0.08% white blood cells and <0.02% red blood cells). Platelets were used for the preparation of cytosol and the lymphocytes were used for DNA isolation.

SULT1A1 Genotype Determination

The polymorphism in the *SULT1A1* gene investigated in these studies consists of a G to A transition that results in an amino acid change (Arg to His, designated *SULT1A1*1* and *SULT1A1*2*, respectively) at residue 213. Detection of the polymorphism was performed according to the method of Ozawa, *et al.* (Ozawa et al., 1998). The region of the *SULT1A1* gene flanking the polymorphic base pair was amplified in a PCR reaction using 5'-GGTTGAGGAGTTGGCTCTGC-3' and 5'-ATGAACTCCTGGGGGACGGT-3' as forward and reverse primers, respectively. The amplification was performed in a 50- μ l volume containing 100 ng of genomic DNA, 200 μ M of each dNTP, 1X PCR buffer (Promega), 1.5 mM $MgCl_2$, 1 μ M forward and reverse primer, 2.5 units Taq polymerase and 500 ng Taq-Start antibody (Clontech, Palo Alto, CA). After initial denaturation at 95°C for 4 minutes, the samples were subjected to 35 cycles of 94°C for 30 sec, 66°C for 30 sec and 72°C for 90 sec, followed by a final extension step of 10 min at 72°C. The resulting PCR product of 281 bp was then subjected to restriction digest with *Hae*III. Bands were resolved on a 3% Metaphor Agarose gel and visualized by ethidium bromide staining and UV transillumination.

Individuals homozygous for *SULT1A1*1* exhibit two bands upon digestion while *SULT1A1*2* homozygotes are not cleaved by this enzyme.

Direct sequencing of the SULT1A1 PCR product

Confirmation of the genotypes was performed by direct sequencing of representative samples using the ³³P Thermo Sequenase Radiolabeled Terminator Cycle Sequencing kit (USB Corp., Cleveland OH) according to the manufacturer's directions. Sequencing reactions were fractionated by electrophoresis on Novex (San Diego, CA) QuickPoint Gels according to the protocol provided by the manufacturer. Sequence bands were detected by autoradiography with overnight exposure of dried gels to Fuji RX X-ray film.

SULT1A1 Enzymatic Activity Assay

Platelet cytosol for the SULT assays was prepared as previously described (Frame et al., 1999), using supernatant that was subjected to 1 hour of 100,000 x g ultracentrifugation at 4°C. Protein determinations were performed with the BioRad Protein Assay kit (Hercules, CA) according to the Bradford Method using bovine serum albumin as a standard. Platelet cytosols were assayed for sulfotransferase activity using a simple colorimetric procedure as described by Mulder (Mulder et al., 1977) with the modifications made by Frame, *et al.* (Frame et al., 1997; Frame et al., 1999). Activity was reported as nmol/min/mg protein.

N-OH-ABP and N-OH-PhIP sulfotransferase activity assays

The assays were carried out as described by Kadlubar, *et al.* (Kadlubar et al., 1976) with the modifications made by Chou, *et al.* (Chou et al., 1995). Activity was reported as pmol bound/mg DNA/20 min.

Statistical Analysis

Because there were no statistically significant differences in phenotype between cases and controls in either the breast or colorectal cancer studies, all participants with both genotyping and phenotyping data were included in these analyses (n=279). Phenotypic differences by race and gender were evaluated using Student's t-test of means, and non-parametric correlations between genotype and phenotype were evaluated using Spearman's rho. Associations between genotype and phenotype were evaluated using analysis of variance, with phenotype as a continuous variable. These analyses were performed for the entire data set, and separately by gender and race. Finally, linear regression was used with the natural log of phenotype as the dependent variable to determine the impact of age, race, gender and SULT1A1 genotype on phenotypic activity.

RESULTS

SULT1A1 Genotyping

Genotyping was performed on DNA from 279 participants; demographic characteristics of the study population are shown in Table 1. The *SULT1A1* G to A transition removes the restriction site for the endonuclease *HaeIII*. As shown in Figure 1, individuals homozygous for the *SULT1A1**2 allele do not have the *HaeIII* restriction site and consequently, the PCR product is not cleaved (Fig.1, lane 1). The PCR product from individuals homozygous for *SULT1A1**1, however, is cleaved by the enzyme, generating two fragments of approximately 100 and 181 base pairs (Fig 1, Lane 2). Enzymatic digestion of the PCR product from heterozygotes (*SULT1A1**1/*2) generates one band of 281 base pairs, along with the 100 and 181 base pair fragments (Fig. 1, lane 3). Direct sequencing of the PCR product was performed on representative samples to confirm the genotyping results and to resolve ambiguous results (data not shown). When cases were excluded from the analysis and only controls were considered (n = 211), distributions were similar to that for cases and controls combined. *SULT1A1* allele distributions are shown in Table 2 separately for Caucasians (n=240) and African-Americans (n=40). Among Caucasians, 18% of the participants were homozygous for *SULT1A1**2 alleles. Although the group size was small, only 10% (n=4) of African-Americans had this genotype.

Sulfotransferase Phenotyping

Platelet cytosols from study participants were analyzed for SULT activity colorimetrically as described in "Materials and Methods". The probit plot shown in Figure 2 demonstrates the distribution of SULT activity toward 2-naphthol in the study

population. Significant ($p=0.001$) differences in activity were noted between men and women. The mean activity level for women was 1.28 (standard deviation [SD]=.90) and for men, 0.94 (SD=.98)

SULT1A1 Phenotype-Genotype Correlation

The correlation between mean levels of SULT activity and SULT1A1 genotype was 0.29, indicating that genotype predicts less than one-third of SULT1A1 phenotype. Using Oneway Analysis of Variance, levels of SULT activity were evaluated by genotype among all participants and separately by race and gender (Table 3). Overall, with each contributing variant allele, there were decreases in levels of activity.. When stratifying by gender and race, differences remained in all subgroups, although they were of borderline significance in males. Tukey's Post hoc tests revealed that differences were significant primarily between those homozygous for the variant alleles and heterozygotes as well as homozygotes for SULT1A1*1/*1. Although in all strata, heterozygotes had lower activity than those homozygous for the common alleles, there were no significant differences in activity between these groups. In a linear regression model, only gender and SULT1A1 genotype significantly impacted phenotypic activity, with gender having the highest β coefficient (Table 4).

N-OH-ABP and N-OH-PhIP sulfotransferase activity assays

Recombinant SULT1A1*1 and SULT1A1*2 were investigated for their relative abilities to catalyze the binding of *N*-OH-ABP and *N*-OH-PhIP to calf thymus DNA. As shown in Table 5, incubations containing equivalent amounts of expressed SULT1A1*1 and SULT1A1*2 showed that the latter had 10-25% of the activity of the wild type toward catalyzing the PAPS-dependent DNA binding of *N*-OH-ABP and *N*-OH-PhIP.

DISCUSSION

Sulfotransferase activity in platelets has been studied extensively by many investigators. Platelets express both SULT1A1 and SULT1A3, and in the case of SULT1A1, the activity correlates to that found in other tissues such as the liver, intestine and brain (Campbell and Weinshilboum, 1986; Sundaram et al., 1989; Young et al., 1985). This correlation, and the easily accessible nature of platelets, has led to their use in population studies. There is substantial interindividual variability in platelet activities; however, it has been reported that there is little intraindividual variability (Anderson and Jackson, 1984; Frame et al., 1999). In contrast to these observations, others have found that platelet SULT activity can be influenced by season (Marazziti et al., 1995). These investigators also found a gender difference in the seasonality of activity (Marazziti et al., 1998). SULT1A1 activity has also been shown to differ by ethnicity (Anderson and Jackson, 1984; Anderson et al., 1988; Kadlubar et al., 1992), with African-Americans having higher SULT activity than their Caucasian counterparts.

In addition to catalyzing the transfer of the sulfonyl group from PAPS to a phenol acceptor substrate, SULT1A1 is also capable of transferring the sulfonyl group between two phenols under neutral or acidic conditions (Mulder et al., 1977). Incubation of platelet cytosol with both 2-naphthol and p-nitrophenyl sulfate in the presence of micromolar amounts of PAPS generates 2-naphthyl sulfate and p-nitrophenol (PNP). PNP can be quantified colorimetrically by changes in absorbance at 405 nm under alkaline conditions. Generation of PNP has been shown to directly correlate with the formation of 2-naphthyl sulfate (Mulder et al., 1977). While SULT1A1 and SULT1A3 are

both capable of sulfating 2-naphthol, the K_m for *SULT1A3* requires much higher substrate concentrations and does not contribute to PNP formation in this assay (Frame et al., 1999). Therefore, it is judged that, with this particular assay, the activity measured is due solely to *SULT1A1*.

Inheritance studies performed in the late 1980's indicated that platelet *SULT* activity was influenced by genetic polymorphisms (Price et al., 1988; Price et al., 1989) but at that time, the molecular basis of the polymorphisms was unknown. Recently, polymorphisms in *SULT1A1* have been identified (Ozawa et al., 1998; Raftogianis et al., 1997). The variant, *SULT1A1*2*, has been associated with low *SULT1A1* activity and thermostability (Raftogianis et al., 1997), although the number of samples with phenotype data available was small.

In this study, we sought to demonstrate a correlation between genotype and platelet phenotype using a microtiter plate assay for platelet *SULT1A1* activity, along with genotyping for the *SULT1A1*1/1A1*2* polymorphism. The allele frequencies were slightly different from those published previously (Coughtrie et al., 1999; Ozawa et al., 1998; Raftogianis et al., 1997), possibly due to numbers genotyped and the nature of the population surveyed. A distinct difference in the allele frequency between African-Americans and Caucasians was observed, with African-Americans being less likely to be homozygous for the *SULT1A1*2* allele (10% of African-Americans compared to 18% Caucasians). This is in contrast to the allele frequencies demonstrated by Coughtrie, et al. who found no statistically significant differences in allele frequencies between a Nigerian and Caucasian population (Coughtrie et al., 1999). This disparity is possibly due to the intrinsic differences between the populations studied (Nigerian vs. African-

American). When phenotype was compared in this study, however, African-Americans consistently exhibited higher activity levels for each genotype than Caucasians (Table 6). This is in agreement with findings by other investigators who have demonstrated higher SULT activity in platelets from African-Americans vs. Caucasians. When African-American males were compared to Caucasian males, overall activity was higher, thus excluding the possibility that the data reflected gender differences.

There was also a difference in SULT1A1 activity between males and females in this study, with females having significantly higher levels than males. The mechanism of the elevation in SULT1A1 activity in females has not been elucidated. Hormonal regulation of another member of the phenol sulfotransferase subfamily, SULT1E, by progesterone has been demonstrated (Falany and Falany, 1996). Therefore, it is possible that the difference in activity between males and females could have a hormonal basis.

There was a dose-dependent effect of genotype on platelet SULT phenotype throughout this study. Individuals possessing the *SULT1A1**2/*2 allele consistently displayed lower platelet activity, while heterozygous individuals tended to have intermediate activity and those homozygous for *SULT1A1**1 exhibited the highest overall SULT activity. The biological role of sulfation by platelet isoforms is unknown at the present time. However, the correlation of platelet SULT1A1 activity with the activity in other tissues provides the opportunity to examine the relationship between genetic polymorphisms and phenotype independent of the contribution of other SULT isoforms to activity levels. This study demonstrates a statistically significant correlation between genotype and platelet phenotype. However, analysis of variance indicates that this

genotype accounts for less than 30% of the phenotypic variation observed. Moreover, probit analysis of each genotype indicates that the stratified phenotype is not normally distributed and that there are likely other genetic determinants of SULT1A1 activity.

Studies using recombinant *SULT1A1* allelic variants revealed that the wild type, SULT1A1*1 catalyzed the binding of proximate carcinogens much more efficiently than did the SULT1A1*2 variant. This is in agreement with the proposed role of SULT1A1 in the activation of heterocyclic amines to DNA binding species, one of the steps considered to be involved in tumor formation. Given the potential role of SULT1A1 in chemical carcinogenesis, further investigation of this genotype-phenotype interaction in the context of a case-control study is underway in our laboratory.

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Table 1. Demographics of the Study Population

Age (mean)	60 ± 11
Gender	
Male	96 (34%)
Female	183 (66%)
Race	
Caucasian	239 (86%)
African-American	40 (14%)

Table 2. *SULT1A1* Genotypes in Caucasian And African-American Populations

SULT1A1 alleles	Caucasian	African American
	N (%)	N (%)
SULT1A1*1/1A1*1	109 (46)	23 (58)
SULT1A1*1/1A1*2	87 (36)	13 (32)
SULT1A1*2/1A1*2	43 (18)	4 (10)
Allele Frequencies		
SULT1A1*1	0.64	0.74
SULT1A1*2	0.36	0.26

Table 3. Relationship between Mean Sulfotransferase Activity and SULT1A1 Genotype

	SULT1A1*1/1*1	SULT1A1*1/1*2	SULT1A1*2/1*2
SULT1A1 activity ^a	n= 132	n= 100	N = 47
(all data) p <0.001	1.40 ± 1.06 ^b n=92	1.18 ± 0.78 n=63	0.74 ± 0.48 n=28
(females) p=0.002	1.44 ± 0.99	1.34 ± 0.72	0.78 ± 0.40
(males) p=0.06	n=40	n=40	n=20
	1.32 ± 1.22 n=109	0.91 ± 0.81 n=87	0.68 ± 0.58 n=43
(Caucasians) p=0.001	1.36 ± 1.12	1.12 ± 0.74	0.76 ± 0.49
	n=23	n=13	n=4
(African-Americans)	1.60 ± 0.75	1.58 ± 0.93	0.53 ± 0.34
p=0.05			

^aOne-way ANOVA

^bActivity units are nmol/min/mg protein ± standard deviation

Table 4. Predictors of SULT1A1 Phenotype^a

Variable	β coefficient	p value
Age	.0028428	0.61
Race	.3282531	0.06
Gender	.5834602	0.000
SULT1A1 Genotype (*1/*2)	-.0136802	0.92
SULT1A1 Genotype (*2/*2)	-.4673483	0.011

^a Using a linear regression model, with natural log of phenotype as dependent variable.

Table 5. SULT1A1-catalyzed DNA binding Activity in Relation to Genotype

Substrate	SULT1A1 variant	DNA binding Activity ^a (pmol bound/mg DNA/20 min)
N-OH-ABP	SULT1A1*1	5.4
	SULT1A1*2	0.4
N-OH-PhIP	SULT1A1*1	4.6
	SULT1A1*2	1.8

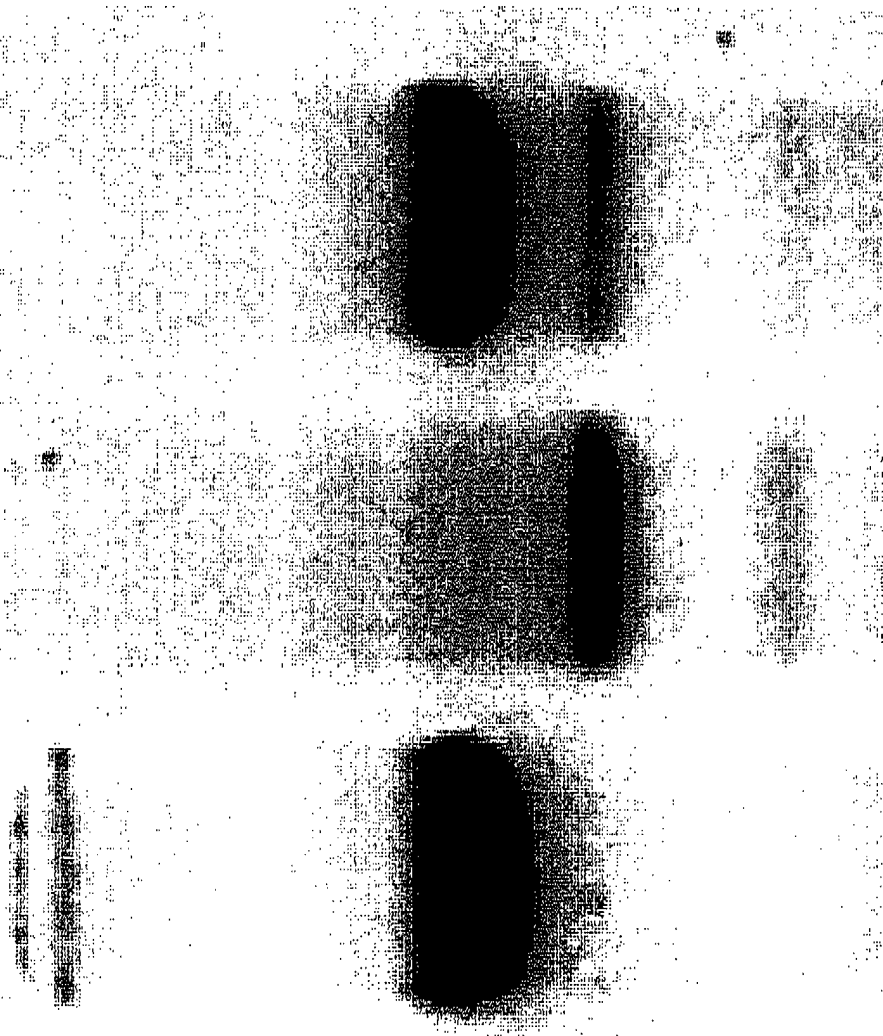
^a An average of duplicate determinations that were within 10% of each other.

FIGURE LEGENDS

- Figure 1. Detection of *SULT1A1**1/*2 alleles by RFLP analysis. Specific PCR product was generated and digested with *Hae*II as described in Materials and Methods. Lane 1: *SULT1A1**2/1*2; Lane 2: *SULT1A1**1/1*2; Lane 3: *SULT1A1**1/1*1.
- Figure 2: Probit plot analysis of platelet *SULT1A1* activity in the study population. The point used as the breakpoint for slow *SULT* activity is indicated on the graph.
- Figure 3: Genotype dependent *SULT1A1* activity in platelet cytosol. Activity was assayed as described in Materials and Methods. The mean activity for each genotype was calculated and plotted.

400 bp --
300 bp --
200 bp --

100 bp --



1

2

3

